Survey of Brevibacillus laterosporus insecticidal protein genes and virulence factors

Survey of *Brevibacillus laterosporus* insecticidal protein genes and virulence factors

Maria Giovanna Marche[a,b], Salvatore Camiolo[a], Andrea Porceddu[a], Luca Ruiu[a,b*]

[a] *Dipartimento di Agraria, University of Sassari, via E. De Nicola, 07100 Sassari, Italy.*

[b] *Bioecopest Srl, Technology Park of Sardinia, Tramariglio, 07041 Alghero, Italy.*

*Corresponding author: Luca Ruiu, Dipartimento di Agraria, University of Sassari, via E. De Nicola, 07100 Sassari, Italy.

Tel.: +39 079229326

E-mail address: lucaruiu@uniss.it

Abstract

The pathogenic action of the bacterium *Brevibacillus laterosporus* against invertebrates involves a toxin-mediated mechanism. Several studies, conducted with specific strains against diverse targets, suggested the implication of different toxins. Recent genome sequencing and annotation of some insecticidal strains revealed several putative virulence factors highly conserved in this species. After determining the pathogenicity of strain UNISS 18 against different Lepidopteran and Dipteran larvae, in this study we have investigated the actual expression of genes encoding for enzymes (i.e., chitinases, proteases), toxins, and other virulence factors, either *in vitro* and *in vivo* at the transcriptional level. Selected genes encode for two chitinases, a collagenase-like protease, a GlcNAc-binding protein, two protective antigen proteins, a bacillolysin, a thermophilic serine proteinase, two spore surface proteins, an insecticidal toxin homologous to Cry75Aa. All target genes were well expressed in pure bacterial cultures with significant differences between bacterial growth phases. Their expression level was generally enhanced in the bacterial population developing in the insect body cavity, compared with pure culture. The expression of certain genes increased substantially over time after insect inoculation. These results support a complex mechanism of action leveraging a variety of available virulence factors, and can also explain the ability of this bacterial species to act against diverse invertebrate targets.

Keywords: bioinsecticide; entomopathogenic bacteria; pathogenesis; toxins; gene expression.

1. Introduction

The growing demand for food quality and safety, the importance of environment protection and the modern legislative frameworks foster the use of environmentally friendly pesticidal products to protect crops, animal and human from pests and parasites (Villaverde et al., 2016). Accordingly, the interest in the discovery and development of new active substances, including bio-based compounds produced by microorganisms is significantly increasing (Lacey et al., 2015). Among these, a main role is played by insecticidal toxins of entomopathogenic bacteria, like the crystal (Cry) proteins synthesized by a variety of *Bacillus thuringiensis* Berliner strains during sporulation that have considerable scientific and industrial importance (Bravo et al., 2011). These toxins normally act in the gut of their host, after being ingested, through specific interaction with insect epithelial membrane receptors, followed by a sequence of events leading to cell membrane disruption and gut paralysis (Adang et al., 2014). Different protein toxins and virulence factors are instead associated with the pathogenicity of other bacterial species acting against insects, whose mode of action has in many cases not been clarified (Ruiu, 2015).

Brevibacillus laterosporus Laubach, is another entomopathogenic bacterium, featuring the production of a canoe-shaped parasporal body (CSPB) attached to one side of the spore and, in the case of some strains, the production of cytoplasmic inclusions containing insecticidal crystal proteins (Zubasheva et al., 2010). Accordingly, a mechanism of action involving the alteration of the insect gut epithelial barrier has been observed (Ruiu. et al., 2012; Ferreira et al., 2016). While the implication of crystal proteins in the insecticidal action has been reported, a significant pathogenicity is also associated with *B. laterosporus* strains lacking parasporal crystals (Ruiu et al., 2007), which supports the involvement of other active substances. Among these, highly conserved putative virulence factors have been identified on the spore surface (Marche et al., 2017). However, bioassays with these proteins were not able to reproduce the full bacterial toxicity, thus suggesting that additional factors are involved in the insecticidal action.

In order to increase our understanding of *B. laterosporus* entomopathogenicity, investigations were conducted on a selection of putative virulence factors that we have identified following the whole genome sequencing and annotation of the insecticidal strain UNISS 18 (Camiolo et al., 2017), characterized by the lack of parasporal crystals (Ruiu et al., 2007). For this purpose the actual expression of genes encoding for enzymes (i.e., chitinases, proteases) and toxins, have been studied either *in vitro* and *in vivo* at the transcriptional level, thus proving their activation during the pathogenic process. Because most of these genes are shared among known *B. laterosporus* genomes, this work provides useful data and information for future studies on different strains of this insect pathogenic species.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The entomopathogenic strain *B. laterosporus* UNISS 18 (= NCIMB 41419) was used in this study. Bacterial cultures were routinely conducted in conical flasks containing Luria–Bertani (LB) broth at 30 °C in an orbital incubator shaking at 180 rpm. Synchronized cultures were obtained as described in Ruiu et al. (2007), using a sequence of LB pre-cultures at the exponential phase. Bacterial growth was checked by phase microscopy and cell cultures were harvested at different growth phases (vegetative, early stationary, sporulation). For this purpose cell fractions were centrifuged at 5000 rpm for 20 min and used fresh in bioassays or stored at -80° for analysis. Living cells used in bioassays were centrifuged at room temperature and resuspended in PBS buffer, while cells and sporangia used for analysis were harvested at 4°C. Living cell counts were routinely performed through plating serial dilutions of bacterial suspensions on LB agar, in order to determine the number of colony forming units (CFU).

2.2. Insect bioassays

Larvae of different insect species were used in preliminary bioassays to test bacterial pathogenicity by injection. These included the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae), the gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae), the lackey moth *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae), the house fly *Musca domestica* L. (Diptera: Muscidae), and the blow fly *Lucilia caesar* L. (Diptera: Calliphoridae). Mass reared *G. mellonella* and *L. caesar* larvae were provided by Microvita (Bologna, Italy). Larvae of *L. dispar* and *M. neustria* were collected in a cork oak forest in central Sardinia (Italy). Larvae of *M. domestica* were provided by the Insect Rearing facilities of the Department of Agriculture of the University of Sassari (Italy).

In order to determine pathogenicity, bacterial suspensions (2 µl) were injected into each surface sterilized larva in between the ventral intersegmental region using a Hamilton syringe. Larvae of each insect species were injected a higher (1000 CFU/larva) and a lower (100 CFU/larva) dose of vegetative cells of *B. laterosporus*. Control larvae were injected 2 µl PBS solution. Treated and control larvae were maintained in groups of 10 inside plastic plates on filter paper inside a growth chamber at 25° C and 50% R.H. Insects were inspected every 12 h for two days, and the number of dead larvae was recorded after each time interval.

Experiments were conducted with a four replicated design and were repeated three times with different batches of insects and bacterial preparations.

Further injection experiments following the same design were conducted on *L. caesar* larvae that were subjected to RT qPCR analyses. Haemolymph samples from pools of these larvae (1 pool = 10 larvae) were analyzed to determine the number of *B. laterosporus* colony forming units (CFU) at different time intervals after injection. These experiments involved three independent biological replicates, and each analysis was performed with three technical replicates.

2.3. *Gene selection and sequencing*

A selection of putative *B. laterosporus* genes associated with pathogenicity and virulence against insects, was identified on the genome of strain UNISS 18, as a result of preliminary genome sequencing, assembly and annotation (Camiolo et al., 2017).

Gene selection has taken into account previous reports on the insect pathogenicity of various *B. laterosporus* strains (Ruiu, 2013; Marche et al., 2017) and other entomopathogenic bacteria (Castagnola and Stock, 2014). Selected genes encode for chitinases, spore surface proteins, insecticidal proteins, metalloproteinases and other enzymes.

While the whole genome of *B. laterosporus* UNISS 18 is deposited at DDBJ/ENA/GenBank under the accession no. MBFH00000000, the complete sequences of target genes have been deposited in the GenBank database under the accession numbers provided in Table 1.

The NCBI Basic Local Alignment Search Tool (BLAST) was used for quick sequence alignments between different strains.

2.4. RT qPCR

The expression of selected *B. laterosporus* genes in correspondence of different growth phases was determined by analyzing the level of mRNA transcripts on pure cell suspensions collected at vegetative (12 h), early stationary (24 h), and sporulation (36 h) phases. Similarly, expression of the same genes was determined at the transcriptional level on haemolymph samples from pools of live *L. caesar* larvae collected at different time intervals (2,6,12 h) after injection with 1000 CFU per larva.

Total RNA was extracted from cultured bacterial suspensions and from haemolymph pools employing TRIzol® Reagent (Life Technologies) according to manufacturer's protocol (Chomczynski and Sacchi, 1987). RNA was routinely quantified and purity checked using a NanoDrop ND-1000 Spectrophotemeter (Thermo Scientific), before being treated with RQ1 RNase-Free DNase (Promega). An aliquot (1 μg) was then used to synthesize first-strand cDNA employing Random Hexamer Primers (Life Technologies), SuperScript® II Reverse Transcriptase (Life Technologies) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Life Technologies) according to the manufacturers' instructions. Power SYBR® Green PCR Master Mix (Life Technologies) was used to run quantitative PCR reactions on an Applied Biosystems 7900HT Fast Real-Time PCR System according to manufacturer's instructions, with the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing at 57-60 °C for 1 min, and extension at 60 °C for 1 min. Forward and reverse primers listed in Table 1 were designed on target gene sequences using Primer3web (version 4.0.0) (Untergasser et al., 2012). Their PCR efficiency was preliminarily tested by standard curve and dissociation curve analyses (Pfaffl, 2001). The relative abundance of qPCR transcripts was determined as suggested by Livak and Schmittgen (2001) using 16S rRNA gene as *B. laterosporus* internal control gene in the *in-vitro* experiments, and both bacterial 16S rRNA and insect actin as reference genes in the *L. caesar* experiments, for PCR normalization (Marche et al., 2017; Mura and Ruiu, 2017). All experiments involved at least three independent biological replicates, and each analysis was performed with three technical replicates.

2.5. Statistical analysis

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS Institute Inc., 2004).

Overtime mortality data of each insect species were analyzed using repeated measures ANOVA (PROC MIXED), and means were separated using LSMEANS comparison (adjust = Tukey).

Linear regression analyses were used for analyzing the relationship between time and bacterial growth (CFU) into growth media or insect haemolymph.

The relative expression of the target genes was analyzed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Fold changes in gene expression in different experiments were subjected to two-ways ANOVA (factors: gene and time), followed by multiple comparison of means (adjust = Tukey).

3. Results

3.1. Pathogenicity of Brevibacillus laterosporus *against different targets*

The pathogenicity of *B. laterosporus* UNISS 18 against larvae of different insect species was assessed by injecting vegetative cells into their body and determining the mortality level after different time intervals. As shown in Table 2, a significant pathogenicity with 100% mortality achieved within 48 h was observed on all treated species compared with the control (*G. mellonella*: $F_{2,33} = 2821.31$, $P < 0.0001$; *L. dispar*: $F_{2,33} = 2525.36$, $P < 0.0001$; *M. neustria* $F_{2,33} = 2935.36$, $P < 0.0001$; *M. domestica*: $F_{2,33} = 1377.94$, $P < 0.0001$; *L. caesar*: $F_{2,33} = 2184.43$, $P < 0.0001$).

A more rapid time to death was generally observed on Lepidopteran compared with Dipteran species, and a faster pathogenic action was detected at higher dose (*G. mellonella*: $F_{2,66} = 1276.75$, $P < 0.0001$; *L. dispar*: $F_{2,66} = 869.56$, $P < 0.0001$; *M. neustria*; $F_{2,66} = 508.12$, $P < 0.0001$; *M. domestica*: $F_{2,66} = 1139.55$, $P < 0.0001$; *L. caesar*: $F_{2,66} = 924.68$, $P < 0.0001$).

3.2. Time course bacterial growth in the insect body

The over time bacterial growth in the haemocoel of *L. caesar* larvae injected with two different doses of vegetative cells (100 and 1000 CFU) was determined by counting the number of CFU at different time intervals after injection and until insect death. The *B. laterosporus* population was found to grow fast in the insect body and the amount of living bacteria (CFU) was positively correlated with time after injection of either a lower (adjusted R2 = 0.7630, F = 191.0, P < 0.0001) or a higher (adjusted R2 = 0.8855, F = 457.5, P < 0.0001) dose (Fig. 1).

3.3. In-vitro expression of genes related to bacterial pathogenicity and virulence

The relative expression (fold change) of target genes at different *B. laterosporus* stages of growth is shown in Fig. 2. All target genes were well expressed in pure culture and the relative level of expression was significantly affected by the gene ($F_{10,264} = 10.99$, $P < 0.0001$) and the bacterial growth phase ($F_{2,264} = 11.99$, $P < 0.0001$). A significant interaction gene x time was also observed ($F_{20,264} = 5.71$, $P < 0.0001$). Significantly higher expression was detected for collagenase-like protease (*prtC*), insecticidal toxin (*mtx*), bacillolysin (*bl18*) and spore surface protein (*cpbA*) genes (exponential vegetative phase: $F_{10,88} = 42.13$, $P < 0.0001$; early stationary phase: $F_{10,88} = 7.73$, $P < 0.0001$; sporulation phase: $F_{10,88} = 6.80$, $P < 0.0001$). Collagenase-like protease (*prtC*) and insecticidal toxin (*mtx*) genes achieved the highest expression level during the vegetative phase, while bacillolysin (*bl18*) gene was highly expressed during both vegetative and sporulation phases. The expression of spore surface protein A (*cpbA*) gene increased gradually over time achieving a maximum at sporulation.

3.4. In-vivo time-course expression of genes related to bacterial pathogenicity and virulence

The relative expression (fold change) of different target genes in blow fly larvae, at progressive time intervals (2, 6 and 12 h) after injection of *B. laterosporus* vegetative cells, is shown in Fig. 3.

The relative level of expression in the insect haemolymph was significantly affected by the gene ($F_{10,264}$ = 28.12, P < 0.0001) and time after inoculum injection ($F_{2,264}$ = 34.25, P < 0.0001). The interaction gene x time was also significant ($F_{20,264}$ = 27.59, P < 0.0001).

For all genes a significant increase in their expression level was achieved at 12 h after injection (*chiA*: $F_{2,24}$ = 23.25, P < 0.0001; *pa1*: $F_{2,24}$ = 7.35; P = 0.0032; *gbp*: $F_{2,24}$ = 6.27; P = 0.0064; *chiD*: $F_{2,24}$ = 18.28; P < 0.0001; *prtC*: $F_{2,24}$ = 13.64; P < 0.0001; *mtx*: $F_{2,24}$ = 14.52; P < 0.0001; *pa2*: $F_{2,24}$ = 21.87; P < 0.0001; *bl18*: $F_{2,24}$ = 15.75; P < 0.0001; *tsp*: $F_{2,24}$ = 43.56; P < 0.0001; *cpbA*: $F_{2,24}$ = 28.22; P < 0.0001; *cpbB*: $F_{2,24}$ = 11.76; P = 0.0003). A mixture of bacterial growth phases, including vegetative cells and early stage sporangia were observed in the haemolymph of these insect samples by phase microscopy. A higher expression level was detected for spore surface protein A (*cpbA*) gene, followed by bacillolysin (*bl18*), chitinase A (*chiA*), chitodextrinase (*chiD*), collagenase-like protease (*prtC*), and insecticidal toxin (*mtx*), that were significantly more expressed than other target genes (Fig. 3).

4. Discussion

The pathogenic properties of *B. laterosporus* against invertebrates, including insects, nematodes and mollusks, are documented by a growing scientific literature (Ruiu, 2013).

From the very first observations, significant differences between strains of this bacterial species emerged, thus supporting the implication of diverse and specific virulence factors against different hosts.

Our preliminary bioassays revealed the pathogenicity of *B. laterosporus* UNISS 18 against a variety of insect targets in different orders (Lepidoptera and Diptera), which is in line with the ability of this species to act against diverse invertebrate targets (Ruiu, 2013; Marche et al., 2016). A rapid growth of the bacterial population inoculated into the insect haemocoel was also observed, highlighting the ability of this insect pathogen to rapidly overcome the host defense mechanisms leading to lethal septicemia. However, when the bacterium is ingested by the host, it needs to cross the intestinal barrier by opening a breach in the peritrophic matrix and disrupting the intestinal wall, before being able to spread in the body cavity. This complex process, could be achieved by the combined or sequential action of different bacterial toxins and virulence factors, among which a main role is likely to be played by chitinases and proteases dissolving chitin and glycoprotein physical barriers (Castagnola and Stock, 2014), and by pore-forming toxins, such as the well known crystal (Cry) proteins, causing damage to the epithelial cells (Jurat-Fuentes and Crickmore, 2017.). Such destructive action in the midgut of insect ingesting *B. laterosporus* has already been documented (Ruiu et al., 2012; Ferreira et al., 2016), while the role of specific virulence factors at different stages of the pathogenic process has not been clarified. Several studies employing different bacterial strains against diverse targets, showed that most of the insecticidal action is associated with bacterial preparations harvested at the stationary and sporulation growth phases. Accordingly, as a result of the first studies on the interaction between *B. laterosporus* and insects, a higher insecticidal activity was observed after the administration of late vegetative cells or sporangia (Favret and Yousten, 1985; Oliveira et al., 2004). The production of insecticidal crystal proteins during sporulation was thereafter reported, thus supporting the implication of parasporal bodies in the insecticidal action of some strains (Zubasheva et al., 2010). The increase in virulence of bacterial preparations harvested at successive time intervals during growth in artificial media was confirmed in time-course experiments employing synchronised cultures of *B. laterosporus* strain UNISS 18 to investigate the involvement of spore surface proteins in the insecticidal action against the house fly (Marche et al., 2017). More recently, novel Cry proteins exhibiting inhibitory activity against coleoptera and lepidoptera have been identified in some *B. laterosporus* strains (Bowen et al., 2017). Nevertheless, other insect toxins like the insecticidal proteins produced during the vegetative phase (VIP) by certain strains were found to act against Coleoptera (Warren, 1997).

Data deriving from recent genome sequencing and annotation of different *B. laterosporus* strains (Djukic et al., 2011; Camiolo et al., 2017) suggested that different strains of these species share a variety of putative toxins, enzymes and virulence factors. However, such genomic studies did not provide information on the actual expression of such bacterial virulence genes. Our study showed that selected genes were well expressed by strain UNISS 18 during bacterial growth in pure culture and during pathogenesis. These target genes encode for two chitinases (ChiA, and ChiD), a collagenase-like protease (PrtC) and a GlcNAc-binding protein (Gbp), that can participate in the degradation of chitin (Prasanna et al., 2013); two protective antigen proteins (PA1 and PA2) possibly implied in the translocation of other toxins and virulence factors into the cell (Barth et al., 2004); a bacillolysin (Bl18) that is common in many *Bacillus* species (Rabideau et al., 2015); a thermophilic serine proteinase (Tsp) that can be associated with a broad substrate specificity (Harrison and Bonning, 2010), two spore surface proteins (CpbA and CpbB), whose involvement in insect pathogenesis has previously been reported (Marche et al., 2017); an insecticidal toxin (Mtx) showing high homology (99%) with the recently discovered Cry75Aa protein acting against Lepidoptera and Coleoptera (Bowen et al., 2017). Consistently, the implication of entomopathogenic bacteria protein fractions, including a mixture of metalloproteases, peptidases, and other enzymes, in the insecticidal action against diptera has already been reported (Ruiu et al., 2015).

In pure culture, the bacillolysin (*Bl18*) gene was the most expressed during the exponential vegetative phase and was also the most expressed, together with the spore surface protein (*cpbA*) gene, during sporulation. The latter, being related to the production of the typical canoe-shaped parasporal body (CSPB), showed a gradual over time increase in the expression level, achieving a maximum at sporulation (Marche et al., 2017). The insecticidal toxin (*mtx*) gene, was well expressed during all bacterial growth phases, showing a higher level during the vegetative phase. The expression level of collagenase-like protease *prtC* gene was always above most target genes with no differences among bacterial stages of growth. Taken together, these results suggest that a different expression level of these genes during different bacterial stages of growth may explain the different level of toxicity associated with each bacterial phase (Ruiu et al., 2007). With a view of developing a bioinsecticide for field applications (Ruiu et al., 2011 and 2014), knowledge on the synthesis activation of toxins and virulence factors during bacterial growth in batch culture is an important information for appropriate manufacturing procedures (Jackson, 2016).

All target genes were generally more expressed by the bacterial population developing in the insect body compared with pure cultures, and the expression of each gene increased substantially over time after insect inoculation. The spore surface protein (*cpbA*) gene was the most expressed, followed by chitinase (*chiA*, and *chiD*), collagenase-like protease (*prtC*), bacillolysin (*Bl18*), and insecticidal toxin (*mtx*) genes. Such expression enhancement is an indication of a stimulatory effect within the insect body environment where the bacterium can exploit its arsenal of toxins and virulence factors for a rapid growth. In the hypothesis in which the bacterium entered the host orally, the same enzymes and toxins could support its passage through the intestinal barriers to reach the nutrient-rich haemocoel (Mura and Ruiu, 2017). According to this scenario, we can imagine a complex mechanism of action leveraging a variety of available virulence factors. This diversity, support the pathogenic potential of this bacterial species against different invertebrates. Further studies will clarify the specific role of each bacterial virulence factor during pathogenesis.

Acknowledgements

This study was supported by the Italian Ministry of Education, University and Research (MIUR) (PRIN project 2015 “BIOPIC”, 2015BABFCF).

References

Adang, M.J., Crickmore, N., Jurat-Fuentes, J.L., 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action, in: Dhadialla, T.S., Gill, S.S. (Eds.), Advances in Insect Physiology. Academic Press, 47, pp. 39-87.

Barth, H., Aktories, K., Popoff, M.R., Stiles, B.G., 2004. Binary bacterial toxins: Biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. Microbiol. Mol. Biol. Rev. 68, 373-402.

Bowen, D.J., Chay, C.A., Flasinski, S., Yin, Y., 2017. Novel insect inhibitory proteins. Monsanto Technology LLC. US Patent Application No. A120170044568.

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. 41(7), 423-431.

Camiolo, S., Porceddu, A., Ruiu, L., 2017. Genome sequence of *Brevibacillus laterosporus* UNISS 18, a pathogen of mosquitoes and flies. Genome Announc. 5 (21), e00419-17.

Castagnola, A., Stock, S.P., 2014. Common virulence factors and tissue targets of entomopathogenic bacterial for biological control of Lepidopteran pests. Insects 5, 139-166.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Djukic, M., Poehlein, A., Thürmer, A., Daniel, R., 2011. Genome sequence of *Brevibacillus laterosporus* LMG 15441, a pathogen of invertebrates. J. Bacteriol. 193, 5535–5536.

Favret, E.M., Yousten, A.A., 1985. Insecticidal activity of *Bacillus laterosporus*. J. Invertebr. Pathol. 45, 195-203.

Ferreira, V.D.S.B., Barcellos, I.D.S., Carramaschi, I.N., Santos-Mallet, J.R., Queiroz, M.M.C., Zahner, V., 2016. Larvicidal activity and effects on post embrionary development of laboratory reared *Musca domestica* (Linnaeus, 1758) (Diptera: Muscidae), treated with *Brevibacillus laterosporus* (Laubach) spore suspensions. J. Invertebr. Pathol. 137, 54-57.

Harrison, R.L., Bonning, B. C., 2010. Proteases as insecticidal agents. Toxins 2, 935–953.

Jackson, T.A., 2016. Entomopathogenic Bacteria: Mass Production, Formulation, and Quality Control, in: Lacey, L.A. (Ed.), Microbial Control of Insect and Mite Pests: From Theory to Practice. Academic Press (Elsevier), London, UK, pp. 125-139.

Jurat-Fuentes, J.L., Crickmore, N., 2017. Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. J. Invertebr. Pathol. 142, 5-10.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1-41.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Marche, M.G., Mura, M.G., Ruiu, L., 2016. *Brevibacillus laterosporus* inside the insect body: Beneficial resident or pathogenic outsider? J. Invertebr. Pathol. 137, 58–61.

Marche, M.G., Mura, M.E., Falchi, G., Ruiu, L., 2017. Spore surface proteins of *Brevibacillus laterosporus* are involved in insect pathogenesis. Sci. Rep. 7, 43805.

Mura, M.E., Ruiu, L., 2017. *Brevibacillus laterosporus* pathogenesis and local immune response regulation in the house fly midgut. J. Invertebr. Pathol. 145, 55-61.

Oliveira, E.J., Rabinovitch, L., Monnerat, R.G., Passos, L.K., Zahner, V., 2004. Molecular characterization of *Brevibacillus laterosporus* and its potential use in biological control. Appl. Environ. Microbiol. 70, 6657–6664.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Prasanna, L., Eijsink, V.G.H., Meadow, R., Gaseidnes, S., 2013. A novel strain of *Brevibacillus laterosporus* produces chitinases that contribute to its biocontrol potential. Appl. Microbiol. Biotechnol. 97, 1601-1611.

Rabideau, A.E., Liao, X., Akçay, G., Pentelute, B.L., 2015. Translocation of non-canonical polypeptides into cells using protective antigen. Sci. Rep. 5, 11944.

Ruiu, L., 2013. *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. Insects 4, 476-492.

Ruiu, L., 2015. Insect pathogenic bacteria in integrated pest management. Insects 6, 352–367.

Ruiu, L., Falchi, G., Floris, I., Marche, M.G., Mura, M.E., Satta, A., 2015. Pathogenicity and characterization of a novel *Bacillus cereus* sensu lato isolate toxic to the Mediterranean fruit fly *Ceratitis capitata* Wied.. J. Invertebr. Pathol. 126, 71-77.

Ruiu, L., Floris, I., Satta, A., Ellar, D.J., 2007. Toxicity of a *Brevibacillus laterosporus* strain lacking parasporal crystals against *Musca domestica* and *Aedes aegypti*, Biol. Control 43, 136-143.

Ruiu, L., Satta, A., Floris, I., 2011. Comparative applications of azadirachtin and *Brevibacillus laterosporus* based formulations for house fly management experiments in dairy farms. J. Med. Entomol. 48, 345-350.

Ruiu, L., Satta, A., Floris, I., 2012. Observations on house fly larvae midgut ultrastructure after *Brevibacillus laterosporus* ingestion. J. Invertebr. Pathol. 111, 211-216.

Ruiu, L., Satta, A., Floris, I., 2014. Administration of *Brevibacillus laterosporus* spores as a poultry feed additive to inhibit house fly development in feces: A new eco-sustainable concept**.** Poultry Science 93: 519-526.

SAS Institute Inc., 2004. SAS/STAT 9.1, User's guide. SAS Institute Inc., Cary, NC.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40, e115.

Villaverde, J.J., Sandín-España, P., Sevilla-Morán, B., López-Goti, C., Alonso-Prados, J.L., 2016. Biopesticides from natural products: Current development, legislative framework, and future trends. BioResources 11, 5618-5640.

Warren, G.W., 1997. Vegetative insecticidal proteins: Novel proteins for control of corn pests, in: Carozzi, N.B., Koziel, M.G. (Eds.), Advances in Insect Control: The Role of Transgenic Plants. Taylor & Francis; London, UK, pp. 109–121.

Zubasheva, M.V., Ganushkina, L.A., Smirnova, T.A., Azizbekyan, R.R., 2010. Larvicidal activity of crystal-forming strains of *Brevibacillus laterosporus*. Appl. Biochem. Microbiol. 46, 755–762.

Table 1 - *Brevibacillus laterosporus* protein genes used for qPCR analysis

Protein product	Gene ID	Accession Number[a]	Primer sequence		Annealing temperature (°C)
			Sense 5’- 3’	Antisense 5’- 3’	
Chitinase A	*chiA*	MG725829	5’-CGAATACAAACAGGAGCCTAA-3’	5’-GCATCCCAAACAGAAGTGAG-3’	57
Chitodextrinase	*chiD*	MG725830	5’ -AGTCAGATTGGTGATCGTAGC-3’	5’-GCGTTTGCTCTACTAATCCCAC-3’	57
Collagenase-like protease PrtC	*prtC*	MG725831	5’-ATCAAACAAGAGTGGATCGAC-3’	5’-ATTCTACCTTCGAGCCCAC-3’	57
GlcNAc-binding protein A	*gbp*	MG725832	5’ -TGGGGAGATTACAGGAGCA-3’	5’-CCATCGTTAAATGTAGCAATGAG-3’	57
Protective antigen domain protein PA1	*pa1*	MG725833	5'-GCTGTATTGATTTGATAGGTTCC-3'	5'-CTAATTTCACCGAAGAAGGATG-3'	57
Protective antigen domain protein PA2	*pa2*	MG725834	5'-ATCACTCCTCTGCTACGCA-3'	5'-CTGCTAGATTCATTCTCTGGTTG-3'	57
Bacillolysin BL18	*bl18*	MG725835	5’-GTACAAGGCGAGGTAGAGAAT-3’	5’-GTCCAAAGAAGGAGGTTACAT-3’	60
Thermophilic serine proteinase	*tsp*	MG725836	5’-ATAATGAATCCCGACCTGGT-3’	5’-TCGATCAGGATGAGAATCTAG-3’	60
Spore surface protein A	*cpbA*	KY124461.1	5’-GCTTCACACGATCAGCAACC-3’	5’TGTAGGCGGGCAGCTAAAAA3’	60
Spore surface protein B	*cpbB*	KY124462.1	5’-TCACCAAGACACAAAGCCCT -3’	5’-GGGCTTTGTGTCTTGGTGAG-3’	60
Insecticidal toxin MTX	*mtx*	MG725837	5'-ATGGTAGCAGTGGTTTTGACT-3'	5’-CATTAGAAGGAAGTCCAACCG-3’	57

[a] Complete gene sequences were deposited in the GenBank database.

Table 2 - In vivo bacterial pathogenicity

Species	Dose[a] (CFU/larva)	Mortality (%) after injection (mean ± SE)[b]		
		12 h	**24 h**	**48 h**
Galleria mellonella	0	0 a	0 a	0 a
	100	11.7 ± 2.0 b	68.3 ± 2.1 d	100 e
	1000	42.5 ± 2.5 c	100 e	100 e
Lymantria dispar	0	0 a	0 a	0 a
	100	15.0 ± 1.9 b	50.0 ± 2.1 d	100 f
	1000	43.3 ± 1.9 c	85.8 ± 2.3 e	100 f
Malacosoma neustria	0	0 a	0 a	0 a
	100	22.5 ± 2.2 b	59.2 ± 2.3 d	100 f
	1000	47.5 ± 2.2 c	88.3 ± 2.7 e	100 f
Musca domestica	0	0 a	0 a	0 a
	100	5.0 ± 1.6 b	37.5 ± 1.8 d	100 f
	1000	18.3 ± 2.7 c	67.5 ± 2.8 e	100 f
Lucilia caesar	0	0 a	0 a	0 a
	100	6.6 ± 2.5 a	39.2 ± 2.6 b	100 d
	1000	9.2 ± 2.3 a	65.0 ± 2.3 c	100 d

[a] Each larva was injected 2 µl suspension of exponential vegetative cells.
[b] For each species, different letters indicate significantly different means (ANOVA Mixed Proc., Tukey adjusted $P < 0.05$).

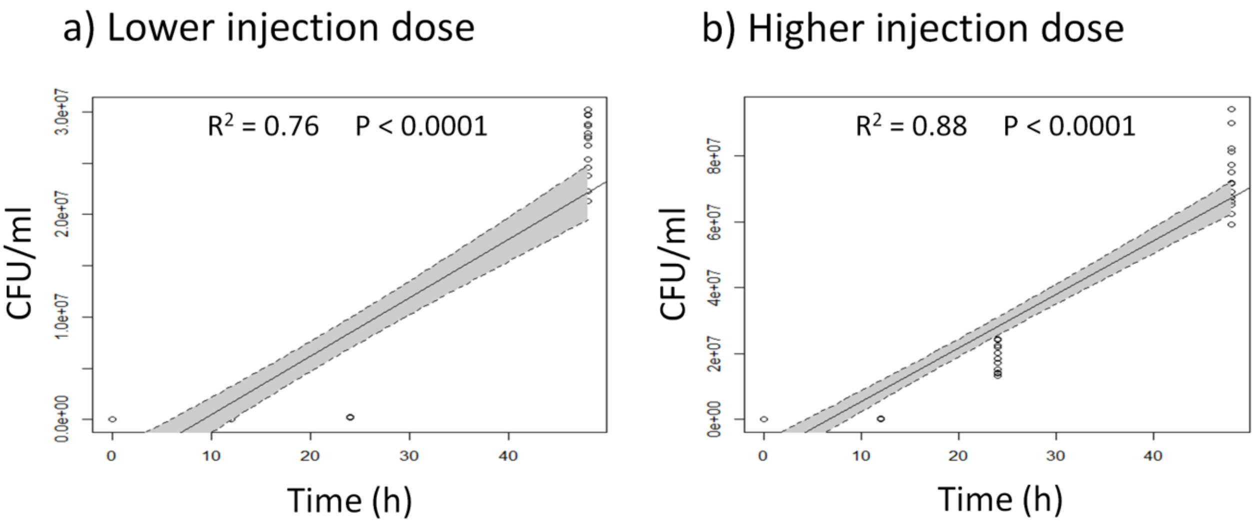
Figure captions

Fig. 1 - Linear regression plots with 95% confidence intervals (shaded areas) showing the predicted relationship between *B. laterosporus* population abundance in the insect haemocoel determined as colony forming units (CFU) and time after injection of a lower (100 CFU) (a) or a higher (1000 CFU) (b) dose of vegetative cells.

Fig. 2 - In-vitro expression of genes related to pathogenicity and virulence in correspondence of different bacterial growth phases. Fold change was obtained using *16S rRNA* transcript abundance at each growth phase for qPCR data normalization. Different letters above bars indicate significantly different means (2-ways ANOVA, Tukey adjusted $P < 0.05$).

Fig. 3 - In-vivo expression of genes related to bacterial pathogenicity and virulence at different time intervals after vegetative cells injection. Fold change was obtained using bacterial *16S rRNA* and insect *actin* transcript abundance at each time interval for qPCR data normalization. Different letters above bars indicate significantly different means (2-ways ANOVA, Tukey adjusted $P < 0.05$).

Fig. 1



a) Lower injection dose
R^2 = 0.76 P < 0.0001
CFU/ml
Time (h)
0.0e+00
1.0e+07
2.0e+07
3.0e+07
0
10
20
30
40
b) Higher injection dose
R^2 = 0.88 P < 0.0001
CFU/ml
Time (h)
0e+00
2e+07
4e+07
6e+07
8e+07
0
10
20
30
40

Fig. 2

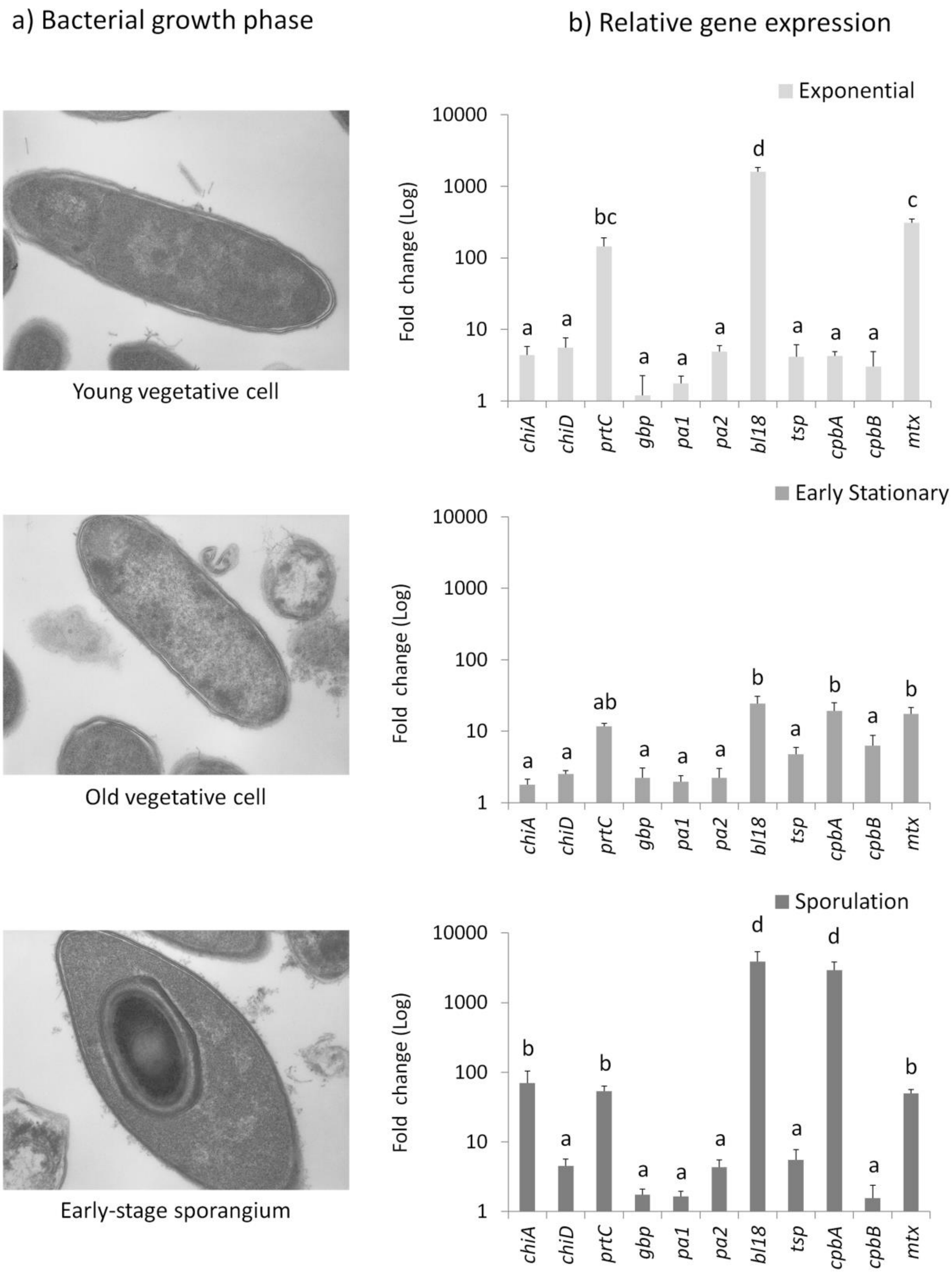
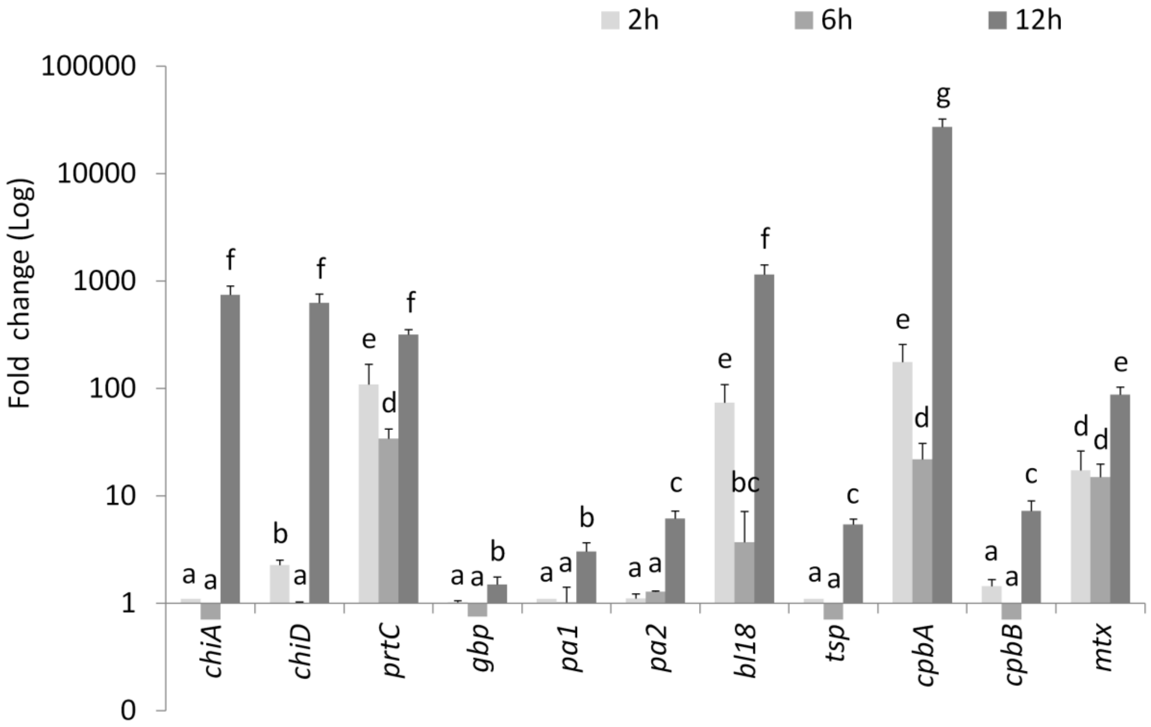

a) Bacterial growth phase
b) Relative gene expression
Young vegetative cell
Old vegetative cell
Early-stage sporangium
Exponential
Early Stationary
Sporulation
Fold change (Log)
10000
1000
100
10
1
chiA
chiD
prtC
gbp
pa1
pa2
bl18
tsp
cpbA
cpbB
mtx

Fig. 3



2h
6h
12h
Fold change (Log)
100000
10000
1000
100
10
1
0
chiA
chiD
prtC
gbp
pa1
pa2
bl18
tsp
cpbA
cpbB
mtx